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The nerve growth factor protein, NGF, has been shown to play a physiologic role in the development and regeneration of the peripheral nervous system, acting on sensory and sympathetic ganglia. In the central nervous system, NGF induces choline acetyltransferase in certain cholinergic regions and spares magnocellular neurons following fimbria transections. NGF has been shown to act in vivo on non-neuronal tissues as a modulator of immune and inflammatory reactivity.

We have demonstrated the presence of receptors to NGF on rat and human mononuclear cells and the specific and saturable binding of NGF to these cells. We have also shown that NGF has activating and mitogenic effects on these cells. Our data is consistent with the hypothesis that NGF effects on tissues are important to differentiation of these tissues. Also, that NGF receptors on different tissues are slightly different structurally although the NGF binding properties are very similar.

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ANNUAL REPORT

Nerve Growth Factor Effects on the Immune System

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I. Introduction

NGF has effects throughout the central (CNS) and peripheral (PNS) nervous system (45,71,94,96). The trophic activities of NGF are essential for the development and survival of regions of CNS cholinergic innervation as well as sensory and sympathetic neurons. The effects of NGF are initiated through its actions on specific cell surface receptors (NGFR) (72,84). NGFR have been found on a variety of neuronal cells, as well as on neuronally-derived culture cell lines (50). For neuronal cells, binding studies with NGF have defined two species of NGFR with equilibrium dissociation constants of approximately 10^{-11}M (Type I, high affinity) and 10^{-9}M (Type II, low affinity) (50,84). Low affinity NGFR have also been demonstrated on rat astroglia and Schwann cells (53). Although the high affinity site is considered the biologically active species for neuronal cells, an interconversion may occur between the low and high affinity sites (52). In addition, the biological consequences of activation of the low affinity site in cells expressing only Type II receptors may be quite different than activational events resulting from Type I interactions. Targets of NGF in the PNS are sensory and sympathetic neurons and the tissues innervated by their fibers. There is a direct correlation between the degree of sympathetic innervation and NGF expression in many organs, including lymphoid tissues (81). This dependence on NGF by sympathetic neurons is shown in experiments involving the addition of NGF and the removal of endogenous NGF with anti-NGF. Injection of exogenous NGF into neonatal and very young rats (3-4 weeks) results in an increase in adrenergic fiber content, as well as elevation of tissue norepinephrine (NE) levels (14). This increased sympathetic response to NGF was not as apparent in rats when treated as adults. Conversely, treatment of both neonatal and adult animals with heterologous antisera against NGF resulted in a time-dependent decrease in sympathetic fiber density and a reduction in NE content of the target organs, including spleen (15,16,88). In the neonate this treatment resulted in a permanent "immunosympathectomy", but in adult animals the effects were transient, with recovery by 3-6 months. Sympathectomy can also be induced by systemic treatment with 6-hydroxydopamine (6OHDA), resulting in extensive reduction in peripheral adrenergic innervations and subsequent functional deficits including changes in immune reactivity (29). It has been found that 6OHDA treatment

also results in an increased synthesis of NGF within the innervated tissues occurring at approximately 2-4 hours of treatment and persisting for 10-14 days (42,97). This increased NGF might contribute to the changes in immune reactivity associated with 6OHDA treatment in adult animals. The interaction between NGF and the SNS does not appear to be unidirectional. In cultured L-M cells and astroglia the addition of catecholamines to the cultures results in growth dependent changes in the synthesis and secretion of NGF (31). NGF also possesses non-neuronal biological activities including the modulation of immune responsiveness (64,86,92,93).

The experimental evidence for a bidirectional flow of information between the nervous system and the immune system is compelling (11,13,78,79). Efferent signals emanate from the CNS where the messages reach the lymphoid compartments either directly through signal molecules generated from neuronal fibers (19,29) or indirectly in the microenvironment via systemically released soluble neuronal factors (29,41,49,67,78). An essential requirement for the communication between the nervous system and cells of the immune system is the presence of ligand-specific cell surface receptors. Studies have confirmed the existence of these receptors for a number of neurosubstances on a diverse population of cells of immune origin (3,24,57). Several reports have also demonstrated functional immunoregulatory responses for many of these neurosubstances both in vitro and in vivo and, depending upon the experimental conditions, these modulatory effects have included enhancement as well as inhibition of the particular response (33,37,40,67). Stead and his colleagues (86) have suggested that within the intact animal, immunoregulatory effects of the nervous system may be a net effect resulting from the interaction of multiple signals on several cell types.

A major role in immune response modulation has been proposed for the adrenergic nervous system (SNS) (12,27,49). Beta-adrenergic receptors are present on T and B lymphocytes and on macrophages in both man and animals (3,38,44). In vitro mitogenic stimulation of lymphocytes by noradrenergic (NA) agonists results in an inhibition of lymphoproliferative activity (39), however, stimulation of cytotoxic T cells (CTL) by NA agonists results in an augmentation of the CTL response (29). Primary and secondary lymphoid organs are extensively innervated by sympathetic NA fibers and some fibers end in direct contact with periarteriolar lymphocytes (29). Pharmacological manipulation of the SNS results in a variety of changes in immune reactivity, depending on the age of the animal and the manipulation paradigm. Short term administration of NA agonists results in down regulation of lymphocytic beta-adrenergic receptor density, decreased lymphoproliferation, and an increased T-dependent IgM response (1). Chemical sympathectomy of neonatal and adult animals with 6OHDA also affects the status of the immune response. Neonatal sympathectomy leads to an increased T-dependent antibody

response, whereas in adult animals treated with 6OHDA, there is an increased response to T-independent antigens without changes in T-dependent responses (59). 6OHDA treatment also affects the phenotypic distribution of lymphocyte subsets (58).

The two proposed main channels in the neuronal control of immune function, direct innervation and circulating neurohormonal factors, are both influenced by endogenous NGF (45,64,72). In addition, NGF can directly influence the activities of cells of the immune system. Treatment of neonatal rats with NGF results in a widespread systemic increase in tissue accumulation of the mast cells (7,86). In contrast, administration of anti-NGF causes a significant reduction in mast cell accumulation (6). Thymectomy or splenectomy reduces the effectiveness of NGF on mast cell increases, suggesting that the accumulation of these cells is at least in part through the effects of NGF on T lymphocytes (6). This hypothesis is supported by recent evidence that the secretion of leukocyte (Eos/Baso; G/M) colony stimulating factors by T-lymphocytes is stimulated by NGF (54). At a cellular level, exposure of mast cells to NGF leads to a calcium-independent degranulation and histamine release (86). NGF is also chemotactic for human neutrophils both in vitro and in vivo (17,32). Mast cells and neutrophils are a major cellular component in acute inflammation. Recent studies have demonstrated that in the early phase of an inflammatory reaction, increased concentrations of NGF (in the nM range) are found within the inflamed tissues, suggesting that NGF may function in a paracrine fashion to modulate in situ immune reactivity. The source of this "inflammatory" NGF remains unknown, but its production by macrophages has been suggested (66), although this source has not been confirmed. The signals resulting in the upregulation of NGF in sites of immune reactivity are unknown, however other secretions by macrophages may be involved. Recently Lindholm and co-workers (48) have found that the monokine interleukin-1 (IL1) can upregulate the expression of NGF mRNA in cultured Schwann cells.

Lymphocytes and macrophages make up the major cellular component of the immune system and are the predominant cells of cellular and humoral immune reactivity. We have shown that lymphocytes and macrophages express specific receptors for NGF (90,61) These receptors were low affinity type and in the rat appear to be present on both T and B lymphocyte subsets (92). The cellular specificity of NGFR bearing human lymphocytes is unknown, but a recent histochemical study by Chesa and her colleagues (23) suggest that they may reside in the B-cell population. We have shown that incubation of splenic mononuclear cells (MC) with NGF resulted in stimulation of proliferative activity, as well as a synergistic response in the presence of T or B mitogens (89). This mitogenic activity was seen in cultures using serum free media, suggesting a direct effect of the ligand (90). Rat thymocyte proliferation was also potentiated by NGF. The

proliferative response of thymocytes is similar to that of MC (93) and more recently this potentiation of MC and thymocytes by NGF has also been found when cocultured with interleukin 2. Antigen-specific responses in vitro, as measured by allogeneic (mouse and rat) mixed lymphocyte reactivity (MLR) and autologous (rat) mixed lymphocyte reactivity (AMLR), is also enhanced by NGF (51,92). One mechanism by which the augmentation of proliferation might occur is the modulation of lymphokine reactivity of the cells by upregulation of interleukin-2 receptors (IL2R) (91). NGF effects on lymphocytic immunity extend to the intact animal. In addition to its effects on mast cells, treatment of rats with NGF to rats results in the accumulation of lymphoblastic cells within the spleen and the circulation (6,86). Also, neonatal NGF therapy followed by immunization results in the augmentation of antibody response to antigenic challenge (2,51).

The evidence for NGFR receptors on immune cells, the influence on functional immune responses of NGF, and its modulation of cellular events associated with inflammation, would suggest that it may play a role as a mediator in the neuro-immune axis. In addition, NGF may also interact with other neurosubstances to influence immune homeostasis. NGF is found throughout the body and is intimately associated with another known modulator of immune reactivity, the SNS. Whether NGF is also a signal generated during psychological events, known to modify immune responsiveness, is at present unknown, however, several studies have found that stress modulates the synthesis of NGF in target tissues (5,8,43).

RESULTS TO DATE

Human PBMC fractions were isolated from healthy donors and indirectly stained for the presence of NGFR (90). The cells were examined by epifluorescence microscopy and approximately 15-25% of the cells were found to express NGFR. NGF binding studies on human PBMC showed specific, reversible, binding of ^{125}I-NGF and Scatchard analysis of the binding gave a single Kd of 10^{-9}M similar to the binding-constants found for rat MC (90).

Since earlier studies showed variable expression of NGFR on splenic MC, we began to investigate whether the NGFR positive cells were segregated within specific lymphocyte subpopulations. Splenic MC were doubly stained for both NGFR and surface markers which delineate specific rat lymphocyte subsets. NGFR were first stained with 192-IgG. The cells were then co-stained with monoclonal antibodies which distinguish T cells (OX-19), T cell subsets (W3/25, T_H; OX-8, $T_{S/C}$) and B cells and macrophages (OX-4, Ia+) and were then examined by fluorescence microscopy. Preliminary sampling revealed NGFR expression on both T- and B-cell populations. Greater than 50% of the Ia+ cells were also NGFR+ indicating a high percentage of B lymphocytes specifically

bind NGF. An interesting parallel is the observation that the highest density of beta-adrenergic receptors also are found on in the B cell population. In addition within the T cell subsets, the majority of cells positive for NGFR resided on the suppressor/cytotoxic cell population ($T_{S/C}$), however a small percentage of the helper cell subset were also positive for NGFR.

Preliminary studies in collaboration with Dr. Scott Whittemore, University of Miami, have revealed the presence of message for NGFR in unfractionated, MC. Splenic MC were collected from animals and after 4 days postimmunization with SRBC. RNA was extracted from the cell preparations and Northern blots prepared using oligonucleotide probes for NGFR and NGF. NGFR-mRNA was detected in both the immunized and unimmunized preparations. Message for NGF was not found in either preparation. Interestingly when the NGFR-mRNA from immunized animals was compared to the unimmunized preparation, a greater amount of specific message was seen in the blot from the immunized animal. These findings confirm the previous observations of the existence of NGFR on rat MC and suggest that in vivo immunological stimulation results in upregulation of the expression of NGFR on responding cell types.

An important extension of the reported findings on the effects of NGF on lymphoproliferation (89) was to examine the possible contributions of the fetal calf serum to the NGF effects. Rat splenic MC cultured in a serum free media (SFM), HL-1 (Ventrex, Portland, ME) showed a similar dose-dependent, positive response to the NGF alone and in the presence of mitogens. Consistently the optimal response of the cells to NGF were seen at lower concentrations of NGF than with serum containing media, suggesting that serum might be inhibitory on NGF. The response of MC in SFM to NGF is also seen when cells are cultured in varying concentrations of IL2.

Mitogen stimulated lymphoproliferation represents a nonspecific correlate of immune reactivity, whereas the allogeneic mixed lymphocyte response (MLR) and the autologous mixed lymphocyte reaction (AMLR) are both in vitro correlates of antigen specific in vivo cell-mediated immune reactivity. Therefore, the allogeneic MLR and the AMLR were utilized as a test of the effects of NGF on antigen specific immune responsiveness. For allogeneic cultures, one-way mixed lymphocyte cultures (MLC) were established using Lewis MC as the responder cell source and irradiated (2500R) Brown-Norway rat MC as the stimulating cell source. NGF augmentation of the MLR was not a dose-dependent phenomenon, but seemed to be a threshold response, occurring only at the highest concentration of NGF tested (10 ug/ml). However, it appears that when plastic adherent cells (macrophages) are removed from the responder population, significant NGF augmentation of the cellular response was observed at lower NGF concentrations. The AMLR, a response to self antigens, has been

proposed to represent cellular reactivity critical for the maintenance of immune tolerance. The effects of NGF on T (responder) vs nonT (stimulator) AMLR activity was examined using Lewis autologous T-cells and non T-cells, prepared by panning on Ig coated plates. In NGF stimulated AMLR, a dose response was seen for all concentrations of the NGF.

We have shown that NGF increases the mitogenic response of mature, differentiated lymphocytes. In order to evaluate the effects of NGF on the proliferative potential of undifferentiated lymphocytes, unfractionated thymocytes were isolated by mechanical disruption from adult male rat thymuses and cultured at various cell densities with NGF alone or together in the presence of mitogens or rat interleukin 2. We found that thymocytes cultured in either RPMI with serum or in SFM, cellular proliferation was stimulated by the addition of NGF alone and that NGF augmented the mitogenic activity of cells stimulated with ConA, PHA and IL2. Subsequent studies have shown that in the augmentation of thymocyte mitogenesis, both NGF and ConA or IL2 must be present at the initiation of the culture. Addition of one of the substance at the initiation of the culture and the other at +24 hours results in control levels of proliferative activity. Four hour NGF pulse seems to be sufficient to stimulate a synergistic mitogenic response of thymocytes. An increase incorporation of tritiated thymidine was observed at 1 μg/ml, however at the higher concentration of NGF tested the stimulation levels were similar to control levels. The reasons for these results are unknown at this time. Initial attempts to visualize NGFR on unfractionated thymocytes were negative, however we were able to demonstrate NGFR on thymocytes using IgG-192 after ConA stimulation. The failure to visualize NGFR may only be a reflection of the low level of expression of NGF receptors on unstimulated cells and subsequent mitogenic stimulation, which leads in part to a differentiation of some of the thymocytes into mature cells, might then upregulate the NGFR to a higher level. Immunohistochemical studies on human thymuses have also failed to reveal positive staining of NGFR (23). Our preliminary dissociation binding studies on solubilized thymocytes isolated from adult rats have revealed specific binding of NGF to isolated membrane preparations with a Kd in the nanomolar range suggesting, as with other immune cells, human and rodent, the presence of a low affinity binding site NGFR on rat thymocytes.

CONCLUSIONS

Lymphocytic function can be modulated by NGF. From our initial receptor studies, NGF activities occur as a result of the interactions of NGF with specific cell subsets within the lymphoid immune system. The specificity of NGF effects on defined immune reactivity needs to be confirmed as does its effects in an

in vivo environment, and therefore we propose to explore these areas. In comparing the mitogen studies using NGF versus adrenergic agonists one sees almost the exact opposite results on proliferative activity, while the antigen specific in vitro reactivities are similar. Given a similar distribution among receptor positive lymphocyte subsets and the close association of NGF with adrenergic innervation, one could speculate on the interactive events of NA and NGF on modulation of specific immune reactivity.

PLANS FOR THE THIRD YEAR

We will do in vitro studies to establish NGF-immunocyte interactions and define the role of NGF in the regulation of immune reactivity in a well defined environment. The distribution of NGFR among specific lymphocyte subsets of MC and PBMC will be examined by flow cytometry.

Our preliminary data shows that NGF modulates the proliferative response of unfractionated thymocytes in the presence of mitogens. However, fluorescence microscopy failed to reveal NGFR on thymocytes until after mitogenic stimulation. We will address the question of the existence and specificity of thymocyte NGFR by examining the NGF binding kinetics of undifferentiated and mitogen differentiated thymocytes. To also examine the possibility of segregation of the NGFR+ cells in either the cortical or medullary compartments, both unfractionated and PNA fractionated thymocytes will be examined.

Previously, we demonstrated that NGF modulates DNA synthesis and proliferation, of rat MC both in the presence and absence of mitogens. We will expand these studies to investigate effects of NGF on the synthesis of RNA and de novo protein synthesis in cultured lymphocytes. In addition our present investigations would suggest that response to NGF is more uniform and dose dependant in serum free media. These results suggest that serum may have an inhibitory effect in the NGF-MC and NGF-thymocyte interactions. We therefore propose to "side by side" compare NGF effects in the presence and absence of serum containing media. We will also investigate the timing of exposure to NGF necessary for the augmentation of lymphoproliferation. Cursory observations suggest that 2-4 hours exposure to NGF may be sufficient for lymphocytic effects. Lastly, since mitogen studies are only a reflection of nonspecific reactivity, we will examine the modulatory effects of NGF on in vitro antigen specific responses and in the vitro generation of antibody producing cells.

Regardless of the in vitro effects of neurosubstances on the modulation of immune reactivity, only the "potential" biological effects of the substance are measured. These experimental observations do not necessarily translate into effects seen in intact animals (or man). Physiologic interactions between elements of the nervous system and the immune system may be the

result of direct ligand-cellular interactions, or may be more complex and may be manifested as the net result of multiple interactions involving several differing substances acting on various cell types. Indeed the overall influence of the NS with the IS might result from a combination of both direct and indirect signal-cell interactions! Since the physiologic responses of multifaceted endogenous factors are difficult to measure, one must turn necessarily to ligand-specific pharmacological models of immunomodulation. Clearly, the presence of NGF in lymphoid tissues, NGFR on immune cells and changes in NGFR after immunization are would suggest that NGF participates in the intact animal in the maintenance or modulation of immune reactivity. Two in vivo studies on the effects of NGF in vivo on immune reactivity (2,51), support this hypothesis. We will begin our in vivo explorations of NGF-immune interactions by measuring the effects of exogenously administered NGF on subsequent immune responsiveness, and by investigating the effects of blocking endogenous NGF by species specific anti-NGF and again testing in vitro and in vivo immune parameters in the animal. Numerous studies on the effects of administration of NGF and anti-NGF on the nervous system and on the immune system confirm that administered NGF and anti-NGF both home to specific NGF responsive tissues, causing either positive or negative NGF reactivity.

Because of the direct neuronal relationship between nerve growth factor and sympathetic innervation, the effects of NGF or anti-NGF on immune function could ultimately result (in part or totally) from their influence on adrenergic innervation of the tissues and its known modulatory effects on the immune system. To begin to address this very important question, we will take advantage of the 6OHDA treatment paradigm which results in the destruction of NA sympathetic nerve terminals and a decrease in total splenic NE content. As has been discussed earlier, 6OHDA therapy of adult animals results in a complete but transient reduction in adrenergic activity in target tissues. However, studies have also shown that this treatment results in a rapid increase in NGF synthesis within the innervated tissues. To what extent the immunoregulatory effects of NGF or anti-NGF might be modified by 6OHDA, will be examined by repeating the studies on the effects of NGF and anti-NGF using animals pretreated with 6OHDA. Although these studies will not answer conclusively the relative contribution of the SNS to the in vivo immunoregulatory properties of NGF, they should address the relationship of the two entities in immune homeostasis.

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